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Diazinon (Trade Name): Chronic Toxicity to, and Brain Acetylcholinesterase Inhibition in, the Sheepshead Minnow, 'Cyprinodon variegatus'

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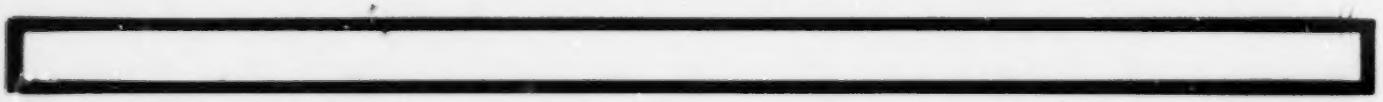

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16. ABSTRACT

The toxicity of Diazinon to sheepshead minnows is described. Although the number of eggs spawned by continuously exposed fish was significantly reduced (α=0.05) in all concentrations, no concentration tested significantly affected parental survival or fertility of eggs, nor were survival and growth of progeny affected in a subsequent 28-day test. Fish exposed to 0.47 µg/liter spawned 22.6 eggs/female-day, or 69% of control production (32.8 eggs/female-day). The number of eggs spawned by fish exposed to 6.5, 3.5, 1.8, and 0.98 µg/liter Diazinon was similar (14.9-17.9 eggs/female-day), averaging 45-55% of production by control fish. Egg production by fish that had been previously exposed to 3.5 µg/liter did not change significantly following depuration for 23 to 31 days. AChE activity varied inversely with exposure concentration, fish in the highest concentration (6.5 µg/liter) averaging 71% inhibition. The concentration of Diazinon measured in adult fish exposed to 1.8, 3.5, and 6.5 µg/liter averaged 169 times the concentration measured in the water. Diazinon was not consistently detected (<0.05 mg/kg) in fish exposed to 0.98 and 0.47 µg/liter. The maximum acceptable toxicant concentration (MATC) for sheepshead minnows continuously exposed to Diazinon, based on reduced fecundity, is <0.47 µg/liter; the application factor (MATC ÷ 96-hour median lethal concentration) is <0.0003.

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Diazinon®: Chronic Toxicity to, and Brain Acetylcholinesterase Inhibition in, the Sheepshead Minnow, *Cyprinodon variegatus*[1]

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Abstract

We investigated the toxicity of Diazinon to sheepshead minnows continuously exposed in flow-through toxicity tests. In the 96-hour acute test, a measured concentration of 1,400 μg/liter was lethal to 50% of the juvenile test animals. In the partial life-cycle test, we also investigated uptake of Diazinon by sheepshead minnows and effect of Diazinon on brain acetylcholinesterase (AChE) activity. Average measured concentrations in the partial life-cycle test were nondetectable (control), 0.47, 0.98, 1.8, 3.5, and 6.5 μg of Diazinon per liter of seawater. Although the number of eggs spawned by continuously exposed fish was significantly reduced ($\alpha = 0.05$) in all concentrations, no concentration tested significantly affected parental survival or fertility of eggs, nor were survival and growth of progeny affected in a subsequent 28-day test. Fish exposed to 0.47 μg/liter spawned 22.6 eggs/female-day, or 69% of control production (32.8 eggs/female-day). The number of eggs spawned by fish exposed to 6.5, 3.5, 1.8, and 0.98 μg/liter Diazinon was similar (14.9–17.9 eggs/female-day), averaging 45–55% of production by control fish. Egg production by fish that had been previously exposed to 3.5 μg/liter did not change significantly following depuration for 23 to 31 days. AChE activity varied inversely with exposure concentration, fish in the highest concentration (6.5 μg/liter) averaging 71% inhibition. The concentration of Diazinon measured in adult fish exposed to 1.8, 3.5, and 6.5 μg/liter averaged 169 times the concentration measured in the water. Diazinon was not consistently detected (<0.05 mg/kg) in fish exposed to 0.98 and 0.47 μg/liter. The maximum acceptable toxicant concentration (MATC) for sheepshead minnows continuously exposed to Diazinon, based on reduced fecundity, is <0.47 μg/liter; the application factor (MATC ÷ 96-hour median lethal concentration) is <0.0003.

The organophosphate insecticide Diazinon[3] is recommended for control of household insects, soil insects, and pests of fruits, vegetables, field crops, forage crops, lawns, and ornamentals (Berg 1977). Estimated United States production in 1972 was 5.44 million kg of active ingredient, 3.18 million of which were used domestically (von Rumker et al. 1974). Usage of organophosphate pesticides such as Diazinon for plant protection will probably increase as additional restrictions are placed on organochlorine pesticides.

The probability of water contamination by Diazinon, as influenced by such factors as degradation, pH, and soil type, was discussed by Allison and Hermanutz (1977). Diazinon has been detected in fresh water at concentrations below 1.0 μg/liter during routine monitoring in the Missouri River at Nebraska City, Nebraska, the Arkansas River at Van Buren, Arkansas, the Canadian River near Whitefield, Oklahoma, and the Gila River above Gillespie Dam, Arizona (Schulze et al. 1973). To our knowledge, Diazinon has not been detected in the marine environment, except during experimental applications (Marganian and Wall 1972). However, the potential exists for contamination of estuarine areas via agricultural and urban runoff.

®Registered trademark, CIBA-GEIGY Corporation. Technical Diazinon (92.6% purity) used was supplied by CIBA-GEIGY Corporation, Agricultural Division, Post Office Box 11422, Greensboro, North Carolina 27409. Mention of trade names or commercial products does not constitute endorsement by the Environmental Protection Agency.

[1] Gulf Breeze Contribution Number 374.

[2] Present address: United States Environmental Protection Agency, 401 M Street S.W., TS 769, Washington, District of Columbia 20460.

[3] 0,0-diethyl-0-(2-isopropyl-4-methyl-pyrimidyl-6)thionophosphate.

Toxicity of Diazinon to fishes has been investigated in acute studies by Weiss (1959, 1961), Weiss and Gakstatter (1964), and Coppage (1971, 1972) and in chronic exposures by Allison (1977) and Allison and Hermanutz (1977). In chronic studies with freshwater fishes continuously exposed to Diazinon, the application factors, obtained by dividing the concentration found "safe" (that is, maximum acceptable toxicant concentration, MATC) by the 96-hour LC50 (median lethal concentration), were probably 10^{-4} for fathead minnows (*Pimephales promelas*), 10^{-4} or possibly lower for brook trout (*Salvelinus fontinalis*), and about 10^{-2} for flagfish (*Jordanella floridae*) (Allison and Hermanutz 1977).

Organophosphate insecticides are known inhibitors of acetylcholinesterase (AChE), the enzyme that hydrolyzes the neurotransmitter, acetylcholine (O'Brien 1960, 1967; Heath 1961). Acetylcholinesterase measurements in fishes have proved valuable in detecting pollution of fresh and estuarine waters by anticholinesterase agents (Williams and Sova 1966; Holland et al. 1967; Carter 1971; Coppage and Duke 1971; Morgan et al. 1973; Livingston and Goodwin 1974; Coppage and Braidech 1976).

We conducted this investigation to determine the chronic effect of Diazinon on the estuarine sheepshead minnow and to compare MATC's and application factors with those previously determined for freshwater fishes. In addition, AChE was monitored to compare Diazinon's chronic effects with the extent of acetylcholinesterase inhibition.

Methods

Acute Toxicity Test

Seawater from Santa Rosa Sound, Florida, was pumped through a sand filter and a 20 μ polypropylene filter to the laboratory constant-head box where it was heated to 30 ± 2 C and aerated. Ambient-salinity water (average, 22.7‰; range, 20 to 25‰) was then delivered intermittently to the six 80-liter exposure aquaria by a dosing apparatus similar to that described by Schimmel et al. (1974). Each cycle of the dosing apparatus (average 173 cycles/day) delivered 1.5 liters of seawater containing 8.8 μl of the carrier solvent (acetone) to the control-with-carrier aquarium and to each aquarium that received one of the five Diazinon concentrations. Nominal exposure concentrations were control, 180, 320, 560, 1,000, and 1,800 μg/liter. Average measured Diazinon concentrations and standard deviations (two samples/exposure concentration) were nondetectable (control), 160 ± 35, 340 ± 163, 640 ± 190, 820 ± 262, and 1,400 ± 141 μg/liter. Water temperature was maintained at 30 ± 2 C by immersing aquaria in a water bath. The photoperiod was 12 hours light and 12 hours darkness. Twenty laboratory-reared juvenile sheepshead minnows averaging 14 mm standard length (SL) were placed in each aquarium. Fish were not fed during the 96-hour test.

Partial Life-Cycle Toxicity Test

The exposure apparatus and method of delivering water and toxicant were the same as in the acute test except that the solvent was triethylene glycol; the apparatus cycled an average of 387 times per day throughout the 108-day exposure and 32-day depuration period; and nominal concentrations were control, 0.63, 1.25, 2.5, 5.0, and 10.0 μg/liter. Average measured Diazinon concentrations and standard deviations in weekly water samples (N = 18 in all cases except 1.8 μg/liter in which N = 19) were nondetectable (control), 0.47 ± 0.13, 0.98 ± 0.26, 1.8 ± 0.51, 3.5 ± 0.75, and 6.5 ± 1.2 μg/liter. Hereafter, all references to Diazinon concentration are to the average measured concentration.

Continuous exposure began with juvenile sheepshead minnows that were collected near Gulf Breeze and acclimated to ambient salinity and 30 C for a minimum of 17 days prior to testing; 110 fish were randomly placed in each of six experimental aquaria. A random sample of 22 fish averaged 22 mm (range, 17.5–27 mm) SL, averaged 0.38 g (range, 0.18–0.68 g) in weight, and, except for 0.034 mg/kg DDE[4], contained no other detectable organochlorine (<0.02 mg/kg) or organophosphate (<0.05 mg/kg) insecticides or polychlorinated biphenyls (<0.02 mg/kg).

After fish attained reproductive size (≥26 mm SL), groups of three females and two males were placed in glass spawning chambers (internal dimensions = 34.5 × 19 × 23 cm high with a water depth of approximately 17 cm) located in each exposure aquarium. The ends and bot-

[4] 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene.

tom of the chambers were covered with 6.4-mm² stainless steel screen that allowed eggs to fall into a removable drawer constructed of glass and 450-μ nylon mesh. Two chambers were built side by side and shared a single drawer divided in half by a partition.

Egg production was monitored during five spawning trials: the first three during continuous exposure; the last two during days 23–31 of depuration from the 3.5-μg/liter exposure. Spawning groups were monitored in both chambers during all spawning trials, except the third, when only one chamber was used. When predation of eggs was suspected or was known to have occurred, or when eggs were lost through human error, spawning data in all aquaria for that day were excluded from the analyses. In trial one, 7 of 10 days' spawning were analyzed; in trial two, 5 of 7 days'; in trial three, 7 of 8 days'; in trial four, 7 of 9 days'; and in trial five, 7 of 7 days'. Eggs were removed daily, counted, and examined microscopically to determine percentage fertility. Fish from spawning trials were sacrificed for chemical analyses.

We investigated the effect of Diazinon on the progeny of adults that had been exposed from 73 to 90 days by (1) rearing embryos in the concentration in which they were spawned, (2) transferring embryos spawned in the control aquarium into each exposure concentration, and (3) transferring embryos from the exposure concentrations in which they were spawned into the control aquarium. We placed 20 embryos in each of two replicate egg cups per treatment. Egg cups (9-cm internal diameter glass Petri dish bottoms to which 10-cm-high, 450-μ nylon mesh tubes were attached) were placed in trays within the exposure aquaria. The trays received water from the dosing apparatus, then delivered it to the aquaria via self-starting siphons that caused the water level within the trays to fluctuate approximately 5 cm. Embryos and hatched fish were observed daily for mortality and signs of poisoning during 28 days of exposure. Fish were fed live brine shrimp (*Artemia* sp.) nauplii. All surviving juveniles were photographed for length measurement.

Chemical Analyses

Throughout the chronic experiment, we periodically monitored Diazinon concentrations, salinity, dissolved oxygen, uptake of Diazinon by fish, and brain AChE activity in fish. Water was analyzed weekly for Diazinon concentration and dissolved oxygen. Oxygen content, determined by the modified Winkler method of Strickland and Parsons (1968), averaged 3.9–4.1 mg/liter in each aquarium. Salinity, determined every 4 hours, averaged 16.5‰ (range, 4 to 26‰). Fish from each aquarium were sampled on exposure days 1, 4, 7, 14, 29, 43, 85, and 108 for brain AChE analyses and Diazinon residue. Brain AChE activity and Diazinon residues were also determined on days 8 and 19 in depurating fish previously exposed to 6.5μg/liter and on day 32 in depurating fish previously exposed to 3.5 μg/liter Diazinon. No detectable (<0.02 mg/kg) organochlorine insecticides or polychlorinated biphenyls were found in fish foods (Biorell®, *Artemia* sp. nauplii, and frozen brine shrimp).

Water samples were analyzed by extracting 1 liter of seawater with two 100-ml volumes of petroleum ether. The combined extracts were concentrated to about 20 ml in a Kuderna-Danish Concentrator on a steam table, then transferred to a nitrogen evaporator and evaporated to the desired volume for analysis by gas-liquid chromatography.

Fish for residue analysis were frozen within 1 hour after being killed in acetone and the brain excised for AChE analysis. Embryos were rinsed with fresh water before freezing. Weighed tissue samples were extracted with three 10-ml volumes of methanol for 30 seconds with the aid of a PT 10-ST Willems Polytron (Brinkman Instruments, Westbury, New York). After each extraction, the tubes were centrifuged and the methanol extract was added to a 250-ml separatory funnel; 100 ml of 2.0% sodium sulfate and 50 ml of petroleum ether were added to the combined extracts. The separatory funnel was shaken for 1 minute. After the solvent phases separated, the lower aqueous phase was drained into a 250-ml beaker and the upper layer was collected in a 25-ml Kuderna-Danish Concentrator tube. The petroleum ether extraction was repeated and the combined extracts concentrated to 20 ml on a steam table. Extracts were transferred to a water bath at 45 C and evaporated to 1 ml under a gentle stream of nitrogen.

The concentrate was transferred to a 200 mm × 9 mm inside diameter (ID) Chromaflex

column (Kontes Glass Company) containing 3.0 g of Florisil topped with 2.0 g of anhydrous sodium sulfate. The column was washed initially with 10 ml of hexane and the Diazinon eluted with 20 ml of 50% (volume/volume) diethyl ether in hexane. The eluates were adjusted to the proper volume for gas-liquid chromatographic analysis.

Determinations were made with a Tracor MT-220 gas-liquid chromatograph with a flame photometric detector operating in the phosphorus mode. The chromatograph was equipped with 182 cm × 2 mm (ID) glass columns packed with 0.75% OV-17, 0.95% OV-210, and 2% OV-101 on 100/120 mesh Gas-Chrom Q. Operating parameters were as follows: oven temperature, 180 C; injector, 225 C; detector, 185 C; nitrogen carrier flow, 40 ml/minute; hydrogen, 200 ml/minute; air, 50 ml/minute; oxygen, 15 ml/minute.

The average recovery of Diazinon was 92% (N = 15, SD = ±4.3) from tissues and 90% (N = 10, SD = ±6.3) from water. All samples were fortified with an internal standard (methyl parathion) prior to analysis to evaluate the integrity of the results. If recovery of the internal standard was less than 65%, the analysis was rejected. Residues were calculated on a wet weight basis without correction for percentage recovery.

Acetylcholinesterase in sheepshead minnow brains was assayed by the pH-stat method of Coppage (1971). For each AChE determination, nine fish brains were divided into pooled samples of three brains each. Percentage AChE inhibition was determined by comparison with mean normal activity of control fish on each day of sampling.

Statistical Analyses

Acute mortality data were analyzed by the probit analysis method of Finney (1971).

Fecundity, fertility, progeny mortality and length, and AChE levels of fish during continuous exposure were analyzed by analysis of variance. AChE levels of depurating fish and control fish were compared by Student's t-test. Duncan's multiple range test was used to determine differences among treatments. In all statistical analyses, the level of significance of $\alpha = 0.05$ was chosen.

Residues were analyzed by nonlinear regression techniques with the two-parameter uptake model developed by Bahner and Oglesby (in press).

TABLE 1.—*Toxicity of Diazinon to juvenile sheepshead minnows in a 96-hour flowing seawater toxicity test at 30 C.*

Exposure concentration, µg/liter				
	Measured			Survival (%)
Nominal	*N*	Mean	SD	
Control with carrier[a]	2	ND[b]		100
180	2	160	±35	100
320	2	340	±163	95
560	2	640	±190	75
1,000	2	820	±262	85
1,800	2	1,400	±141	50

[a] Contained 5.8 microliters acetone per liter of seawater.
[b] ND = not detectable (<0.05 µg/liter).

Acute Toxicity Test

Fifty percent of the sheepshead minnows exposed to the highest Diazinon concentration (1,400 µg/liter) for 96 hours died (Table 1). The calculated 96-hour LC50, based on measured concentrations, is 1,470 µg/liter (95% confidence limits = 1,070 to 3,310 µg/liter) with a chi-square value of 0.54. Test animals in all Diazinon concentrations were visibly affected within 24 hours. A syndrome characteristic of poisoning observed in exposed fish included the following signs: lethargy; abnormal forward extension of pectoral fins; darkened areas on the posterior part of the body; and, when startled, sudden rapid swimming in circles followed by paroxysms of severe muscular contractions.

The acute toxicity of Diazinon to sheepshead minnows is comparable to the 96-hour LC50 values for freshwater fishes exposed to Diazinon under flow-through conditions: 770 µg/liter for brook trout, 7,800 µg/liter for fathead minnows, 1,600 µg/liter for flagfish, and 460 µg/liter for bluegills (Allison and Hermanutz 1977).

Partial Life-Cycle Toxicity Test

Survival and Poisoning of Parental Fish

There was no significant toxicant-related mortality of parental fish; excluding deaths due to sampling and handling, only six fish died during the test.

External signs of poisoning were first ob-

TABLE 2.—*Egg production by sheepshead minnows continuously exposed to measured concentrations of Diazinon in flowing seawater at 30 C. Spawning groups consisted of three females and two males from a given exposure concentration.*

Exposure concentration (μg/liter)	Average number eggs/female-day					Totals	
	Trial I[a]		Trial II[b]		Trial III[a]	Average number eggs/female-day	Fertility (%)
	Group 1	Group 2	Group 1	Group 2	Group 1		
ND[c]	37.8	33.7	33.9	27.7	29.6	32.8	98.1
0.47	16.3	12.7	39.9	21.0	27.7	22.6[d]	97.2
0.98	17.4	19.1	13.6	7.2	21.1	16.4[e]	96.7
1.8	21.0	16.1	13.2	16.9	14.4	16.5[e]	98.1
3.5	30.9	12.0	12.5	17.5	15.0	17.9[e]	97.9
6.5	18.5	17.7	8.7	18.9	10.0	14.9[e]	97.6

[a] Spawning data for 7 days were analyzed.
[b] Spawning data for 5 days were analyzed.
[c] Control with carrier; ND = not detectable (<0.05 μg/liter).
[d] Significantly different from control and from 0.98-, 1.8-, and 6.5-μg/liter exposures ($\alpha = 0.05$).
[e] Significantly different from control ($\alpha = 0.05$).

served on day 39 when parental fish were stressed by being captured and counted. Several fish in 1.8-, 3.5-, and 6.5- μg/liter aquaria exhibited abnormal darkened areas of the body and anterior projection of pectoral fins; a few fish exhibited abnormal lateral curvature or flexure of the body and muscular tetany during capture. These signs disappeared approximately 20 minutes after the fish were returned to the aquaria. When fish were captured for placement in spawning chambers on days 72 and 86, stress was minimized by capturing the fish with as little disturbance as possible. Consequently, the only sign of poisoning observed was in fish exposed to 3.5 and 6.5 μg/liter, some of which exhibited dark areas on their bodies.

Reproduction

The average number of eggs produced per day by spawning groups of three female and two male sheepshead minnows was significantly less in all Diazinon concentrations than the number produced by control fish (Table 2). Egg production in 6.5, 3.5, 1.8, and 0.98 μg/liter was similar (14.9–17.9 eggs/female-day), averaging 45 to 55% of the number produced by control fish. The number produced by fish exposed to 0.47 μg/liter was not significantly different from the number produced by fish exposed to 3.5 μg/liter but was significantly less than that produced by control fish and significantly greater than that produced by fish exposed to 0.98, 1.8, and 6.5 μg/liter Diazinon.

Four groups of fish previously exposed to 3.5 μg/liter were spawned during days 23–31 of depuration in Diazinon-free water to determine if egg production had returned to the level of control fish. Although not illustrated in Table 2, the number of eggs produced by the fish (average 18.5 eggs/female-day) did not differ significantly from the number produced by fish subjected to 3.5 μg/liter during continuous exposure, but remained significantly different from that of control fish during the test. When subjected to chemical analysis following spawning trials (day 32 of depuration), these fish contained no detectable Diazinon (<0.05 mg/kg) and their acetylcholinesterase activity was normal as was the AChE activity in fish previously exposed to 6.5 μg/liter after 19 days depuration. These data suggest that residual effects of this pesticide can occur in fish that have no detectable amounts in their tissue and exhibit no significant depression of AChE. Therefore, monitoring of residues or for depression of AChE activity may fail to detect fish that have been adversely affected by exposure to this and possibly other AChE inhibiting pesticides.

The effect of Diazinon on egg production by fish, during and following exposure, was investigated by Allison (1977). Flagfish (*Jordanella floridae*) were exposed to Diazinon in a continuous life-cycle exposure, as well as in 21-day pulse exposures of selected life stages in which concentrations were increased from zero to maximum in four equal daily increments and maximum concentrations were maintained for 12 days before being reduced to zero by four

TABLE 3.—*Mortality of embryos, fry, and juvenile sheepshead minnows and standard lengths of surviving fish that were continuously exposed for 28 days to measured concentrations of Diazinon. Forty embryos were exposed per treatment. No significant difference ($\alpha = 0.05$) existed between treatments (replicates have been combined).*

Exposure concentration (μg/liter)		Mortality (%)			
Parental fish	Embryos and juvenile fish	Embryos	Fry and juveniles	Combined	Average standard length (mm)
ND[a]	ND	7.5	7.5	15.0	8.8
ND	0.47	7.5	7.5	15.0	6.8
ND	0.98	10.0	2.5	12.5	7.4
ND	1.8	0.0	0.0	0.0	6.6
ND	3.5	0.0	0.0	0.0	7.6
ND	6.5	0.0	15.0	15.0	7.3
0.47	ND	7.5	7.5	15.0	7.9
0.47	0.47	15.0	5.0	20.0	7.9
0.98	ND	2.5	2.5	5.0	7.1
0.98	0.98	5.0	0.0	5.0	7.5
1.8	ND	4.8[b]	0.0	4.8	7.9
1.8	1.8	0.0	0.0	0.0	6.9
3.5	ND	10.0	0.0	10.0	7.5
3.5	3.5	0.0	2.5	2.5	6.2
6.5	ND	2.5	12.5	15.0	8.2
6.5	6.5	2.5	0.0	2.5	6.5

[a] Control with carrier; ND = not detectable (<0.05 μg/liter).
[b] Forty-two embryos.

equal decrements at 2-day intervals. Egg production during the life-cycle exposure was not affected by 240 μg Diazinon/liter. However, temporary but complete inhibition of reproduction occurred in the 21-day pulse exposure of the adult-spawning stage when the concentration reached about 130 μg/liter. Fish exposed to a maximum 12-day peak concentration of 130 μg/liter resumed spawning when the concentration was reduced, but fish exposed to 12-day peak concentrations of 210 and 380 μg/liter did not resume spawning until concentrations were below 130 μg/liter for 1–2 weeks. During a postexposure period (described by Allison as being "from 10 days of spawning immediately following exposure plus 7 days of spawning 1 month after exposure was terminated"), egg production by fish previously exposed during the adult-spawning stage was equal to or greater than that of control fish. Acetylcholinesterase was not monitored, so we cannot compare reproductive impairment and AChE inhibition.

Fertility of eggs in our experiment averaged more than 95% in all treatments and was not significantly different among treatments (Table 2).

Mortality and Growth of Progeny

Neither mortality nor growth of surviving progeny was significantly affected by exposure to Diazinon (Table 3) in our experiment. Mortality of embryos to time of hatching averaged 4.7% (range, 0 to 15%). Mortality of progeny from embryos to juveniles (28 days postfertilization) averaged 8.6% (range, 0 to 20%). Juvenile fish averaged 7.4 mm standard length

TABLE 4.—*Acetylcholinesterase (AChE) activity, expressed at 100× micromoles of acetylcholine hydrolyzed per hour/mg brain tissue, in brains of sheepshead minnows continuously exposed to Diazinon and in exposed fish followed to depurate in Diazinon-free water. Each assay sample consisted of pooled brains from three fish, and three samples were assayed to obtain each datum. When AChE activity was significantly less ($\alpha = 0.05$) than that of controls, percentage inhibition is shown in parentheses.*

Measured concentration (μg/liter)	Exposure								Depuration		
	Day 1	Day 4	Day 7	Day 14	Day 29	Day 43	Day 85[a]	Day 108[a]	Day 8	Day 19	Day 32
ND[b]	168	186	206	192	145	181	196	194	194	183[c,d]	201[d]
0.47	168c	112 (40)	170 (17)	168	128	169	154 (21)	153 (21)			
0.98	187	182	146 (29)	136 (29)	121	131 (28)	127 (35)	102 (47)			
1.8	185	130 (30)	103 (50)	90 (53)	96 (34)	88 (51)	96 (51)	66 (66)			
3.5	142	66 (64)	76 (63)	87 (55)	59 (59)	71 (61)	68 (55)	60 (69)			193[a]
6.5	62 (63)	60 (68)	48 (77)	62 (68)	56 (61)	44 (76)	48 (76)	43 (78)	132 (32)	171[c]	

[a] Fish from reproduction-trial assays.
[b] Control with carrier; ND = not detectable (<0.05 μg/liter).
[c] Only two samples were assayed.
[d] Feral controls.

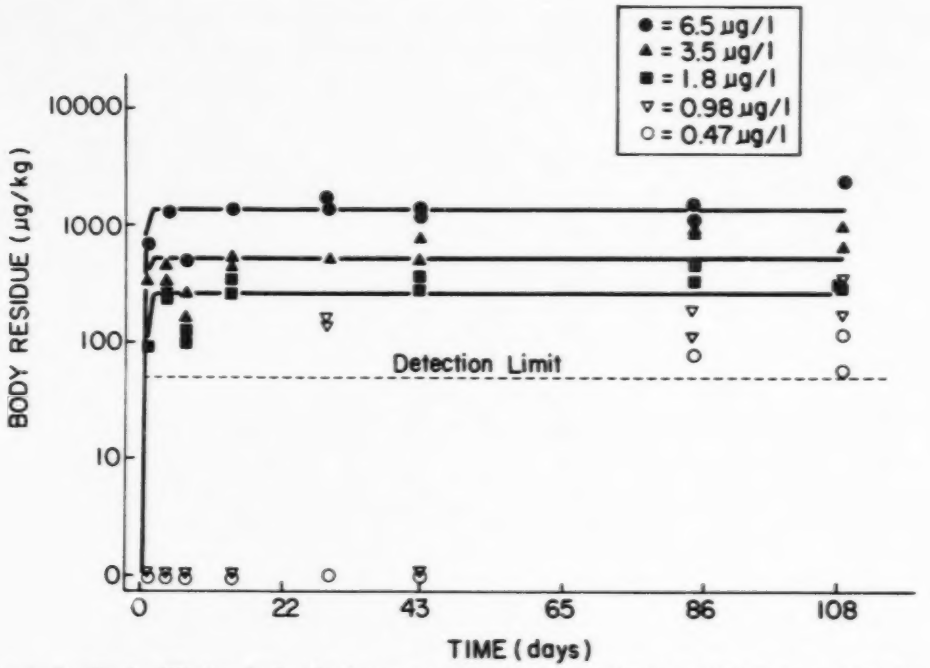

FIGURE 1.—*Uptake of Diazinon by sheepshead minnows (residues in whole body less brain which was removed for AChE assays). Uptake curves were drawn by computer model of Bahner and Oglesby (in press). Nondetectable residues (<0.05 mg/kg) were plotted as zero.*

and 0.009 g. Signs of poisoning observed during parental exposure were not evident in progeny.

Acetylcholinesterase

Acetylcholinesterase activity of parental fish was inversely dependent upon the exposure concentration (Table 4). After 7 days, AChE activity of fish exposed to 0.47 and 0.98 µg Diazinon/liter was consistently less than that of control fish, but statistical analyses of AChE activity of these fish occasionally showed significant inhibition before reproduction studies and consistently significant inhibition in fish assayed following reproduction studies. AChE activity was significantly inhibited by exposure day 4 in fish exposed to 1.8 and 3.5 µg Diazinon/liter and by exposure day 1 in fish exposed to 6.5 µg/liter. Thereafter, AChE activity in these fish remained significantly depressed. Fish exposed to 6.5 µg/liter averaged 71% inhibition, but when transferred to Diazinon-free water, their AChE activity was only 32% inhibited after 8 days and was not significantly different from that of feral controls of similar size by 19 days (no carrier-control fish remained).

The effects of organic phosphorous insecticides on freshwater fish and their subsequent recovery after transfer to uncontaminated water were studied by Weiss (1959, 1961). AChE activity of golden shiners (*Notemigonus crysoleucas*) was approximately 43% inhibited after static exposure to 10 µg Diazinon/liter for 5 hours, but recovered to within the normal range by the eighth day after transfer of exposed fish to Diazinon-free water (Weiss 1959). However, in studies with four species of fish and 10 organic phosphorous insecticides, Weiss (1961) determined that the length of time required for recovery of AChE activity to normal following exposure is dependent on the extent of initial inhibition, the species tested, and the chemical formulation of the insecticide.

In our study, a concentration of 3.5 µg/liter Diazinon produced more than 50% brain AChE inhibition within 96 hours (Table 4) and

TABLE 5.—*Concentrations of Diazinon (mg/kg whole body, less brain) measured in sheepshead minnows continuously exposed to measured concentrations of Diazinon. Fish in the two highest concentrations were allowed to depurate in Diazinon-free seawater.*

Exposure concentration (μg/liter)	Exposure								Depuration		
	Day 1	Day 4	Day 7	Day 14	Day 29	Day 43	Day 85	Day 108	Day 8	Day 19	Day 32
ND[a]	ND[a]	ND	ND	ND	ND	ND	ND	ND			
	ND	ND	ND	ND	ND	ND	ND	ND			
0.47	ND	ND	ND	ND	ND	ND	0.08	0.11			
			ND	ND	ND	ND	0.08	0.05			
0.98	ND	ND	ND	ND	0.14	ND	0.11	0.17			
			ND	ND	0.16		0.19	0.33			
1.8	ND	0.25	0.10	0.26		0.34	0.47	0.31			
		0.27	0.12	0.36		0.30	0.31	0.33			
3.5	0.33	0.45	0.16	0.52	0.51	0.51	1.0	0.65			ND
		0.33	0.26	0.46		0.79	0.96	0.99			ND
6.5	0.70	1.3	0.50	1.4	1.7	1.2	1.1	2.4	ND	ND	
				1.4	1.4	1.4	1.5	2.4	ND	ND	

[a] Control with carrier; ND = not detectable (<0.05 μg/liter in water, <0.05 mg/kg in tissue).

was about 0.0025 times the concentration that produced 50% mortality in 96 hours (Table 1). Therefore, AChE inhibition may have value as an early indicator of chronic poisoning by organophosphate pesticides.

Bioconcentration and Depuration

Uptake of Diazinon by parental sheepshead minnows was rapid, reaching steady state within 4 days in the three highest concentrations (Fig. 1). The concentration reached was dependent on the exposure concentration; bioconcentration factors (concentration in tissues at steady state ÷ concentration measured in water) were 147 for fish exposed to 1.8 μg/liter; 147 for 3.5 μg/liter; and 213 for 6.5 μg/liter. At the two lower concentrations, there were too few data points (Table 5) above the detection limit to permit plotting with the uptake model of Bahner and Oglesby (in press).

Diazinon was depurated (parent compound disappeared) rapidly by parental fish placed in Diazinon-free water. Fish previously exposed to 6.5 μg/liter contained no detectable Diazinon (<0.05 mg/kg) when sampled for the first time on depuration-day 8. Fish previously exposed to 3.5 μg/liter contained no detectable Diazinon when sampled for the first time after 32 days' depuration.

Diazinon was not detected (<0.12 mg/kg) in eggs from fish exposed to 0.47 and 0.98 μg/liter; but 0.14 mg/kg was measured in eggs from fish exposed to 1.8 μg/liter, 0.30 mg/kg in eggs from fish exposed to 3.5 μg/liter, and 0.67 mg/kg in eggs from fish exposed to 6.5 μg/liter. The pesticide burden in eggs had no significant effect on survival of embryos or on survival and growth of fry and juvenile fish when eggs from previously exposed parents were placed in Diazinon-free water (Table 3).

Since Diazinon was not detected in juvenile progeny exposed to 6.5 μg/liter, progeny from lower concentrations were not analyzed. Absence of Diazinon in juvenile fish is attributed to the small sample weight and the correspondingly higher detection limit (0.5 mg/kg).

MATC and Application Factor

The maximum acceptable toxicant concentration (MATC) for sheepshead minnows continuously exposed to Diazinon, based on reduced fecundity at 0.47 μg/liter and higher concentrations, is less than 0.47 μg/liter. For freshwater fish continuously exposed to Diazinon, the MATC's and adverse affects used to determine them are <0.55 μg/liter for brook trout, based on reduced growth of progeny; close to 3.2 μg/liter for fathead minnows, based on reduced hatching success of progeny (Allison and Hermanutz 1977); and between 54 and

88 μg/liter for flagfish, based on reduced hatching success of progeny, although not specifically stated by Allison (1977).

In our experiment, the application factor for sheepshead minnows, obtained by dividing the MATC by the 96-hour LC50 (1,470 μg/liter), is $<3 \times 10^{-4}$ and is similar to those for the freshwater brook trout and fathead minnow. Application factors for freshwater fish continuously exposed to Diazinon are: 10^{-4} or lower for brook trout; probably 10^{-4} for fathead minnows; and about 10^{-2} for flagfish (Allison and Hermanutz 1977). According to the application factor concept of Mount and Stephan (1967), as summarized by Eaton (1970, 1973), the experimentally derived application factor (MATC ÷ 96-hour LC50) is a value that, once determined for any species of fish, can be applied to acute LC50 values to estimate MATC's for other species of fish. In tests with four pesticides, the application factors for the estuarine sheepshead minnow were similar to those for freshwater fishes (Parrish et al. 1978). However, Allison and Hermanutz (1977) caution against the use of single application factor for Diazinon because they found a range of application factors (10^{-2} to 10^{-4}) for different species of freshwater fish.

Conclusions

Our data for sheepshead minnows and those of Allison and Hermanutz (1977) and Allison (1977) for brook trout, fathead minnows, and flagfish demonstrate that fishes are affected by chronic exposures to Diazinon concentrations two to four orders of magnitude less than concentrations lethal to 50% of the test animals in acute toxicity tests. Impaired reproduction in sheepshead minnows and acetylcholinesterase inhibition of 27% occur concurrently during continuous long-term exposure to Diazinon; but reproduction can remain impaired for at least 3 to 4 weeks after fish are placed in clean water, even when their AChE activity is normal and they contain no detectable residues. We, therefore, conclude that the environmental hazard of Diazinon to both freshwater and saltwater fishes is related to its sublethal effects at very low concentrations, effects unlikely to be seen as fish kills and that may not be detected by typical monitoring for Diazinon residues.

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